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Phosphorus Stress Effects on Assimilation of Nitrate[1]

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ABSTRACT

An experiment was conducted to investigate alterations in uptake and assimilation of NO_3^- by phosphorus-stressed plants. Young tobacco plants (*Nicotiana tabacum* [L.], cv NC 2326) growing in solution culture were deprived of an external phosphorus (P) supply for 12 days. On selected days, plants were exposed to $^{15}NO_3^-$ during the 12 hour light period to determine changes in NO_3^- assimilation as the P deficiency progressed. Decreased whole-plant growth was evident after 3 days of P deprivation and became more pronounced with time, but root growth was unaffected until after day 6. Uptake of $^{15}NO_3^-$ per gram root dry weight and translocation of absorbed $^{15}NO_3^-$ out of the root were noticeably restricted in −P plants by day 3, and effects on both increased in severity with time. Whole-plant reduction of $^{15}NO_3^-$ and ^{15}N incorporation into insoluble reduced-N in the shoot decreased after day 3. Although the P limitation was associated with a substantial accumulation of amino acids in the shoot, there was no indication of excessive accumulation of soluble reduced-^{15}N in the shoot during the 12 hour $^{15}NO_3^-$ exposure periods. The results indicate that alterations in NO_3^- transport processes in the root system are the primary initial responses limiting synthesis of shoot protein in P-stressed plants. Elevated amino acid levels evidently are associated with enhanced degradation of protein rather than inhibition of concurrent protein synthesis.

As major nutrient ions, N and P are intimately involved in plant metabolism and growth, and there are numerous points of interaction between N and P dependent processes. It would therefore be expected that in plants deprived of an optimal P supply, assimilation of N could be altered significantly. Experimental results indicate that this may be the case when plants are assimilating nitrogen as NO_3^-. Uptake of NO_3^- g^{-1} of root was decreased by 65% when barley plants were deprived of P (13). Decreases in NO_3^- uptake cm^{-1} of root averaged 58% in a study with P-deficient barley, buckwheat, and rape plants, and the uptake effects preceded large reductions in growth (25). Furthermore, a larger proportion of absorbed NO_3^- accumulated in roots of P-stressed barley (13), implying restricted translocation of NO_3^- out of the root into the xylem. Elevated root concentrations of NO_3^- also were observed in P-stressed soybean plants (7).

In addition to the effects exerted on NO_3^- transport processes in roots, P deficiency may cause a disruption in net synthesis of protein. In studies with a variety of species, P stress was associated with elevated concentrations of amino acids and soluble reduced-N in leaf tissues (7, 20, 21, 28). It is unknown whether the increases in accumulation of amino acids were due to inhibition of protein synthesis or to increased protein degradation.

Available evidence thus indicates that there may be multiple points of regulation in the NO_3^- assimilation pathway when plants experience a P limitation. This research was initiated to investigate the P-stress response further. In an experiment with labeled $^{15}NO_3^-$, an attempt was made to evaluate the relative sensitivities of NO_3^- transport and assimilation processes to a developing P stress, and to determine the extent of inhibition of concurrent protein synthesis.

MATERIALS AND METHODS

Plant Culture

Seed of tobacco (*Nicotiana tabacum* [L.], cv NC 2326) were germinated on a soil mixture in 170 mL plastic pots located in a greenhouse. The seedlings were watered daily (mornings), received one-half strength Hoagland solution twice weekly, and were exposed to natural sunlight only. After 7 weeks, 32 seedlings were selected for uniformity and placed into two 115-L continuous flow, hydroponic culture systems. The culture systems were located in a controlled-environment growth room programmed for 28°C/22°C during the 12/12 h light/dark cycle. A photosynthetic photon flux density of 1100 ± 50 μmol m^{-2} s^{-1} (at canopy height) was provided during the 12-h light period from a combination of high pressure sodium and metal halide lamps.

The temperature of the culture solution was 24 ± 1°C and the solution pH was maintained at 5.8 ± 0.2 by automatic additions of 0.2 N H_2SO_4. Nutrient concentrations in solution were 1.0 mM NO_3^-, 0.1 mM $H_2PO_4^-$, 1.1 mM K^+, 1.0 mM Ca^{2+}, 1.0 mM Mg^{2+}, 2.0 mM SO_4^{-2}, 17 μM B, 5 μM Cl, 3 μM Mn, 0.3 μM Zn, 0.1 μM Cu, 0.04 μM Mo, and 18 μM Fe as ferric diethylenetriamine pentaacetate (Fe-DTPA, CIGA-Geigy Corp).[2] The solutions were changed every 2 d to avoid nutrient depletion effects.

Experimental Conditions

The experiment began on d 8 after transplanting into the hydroponic system. At the beginning of the light period, 16

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[2] Use of trade names does not imply endorsement by the USDA or the North Carolina Agricultural Research Service of the products named, nor criticism of similar ones not mentioned.

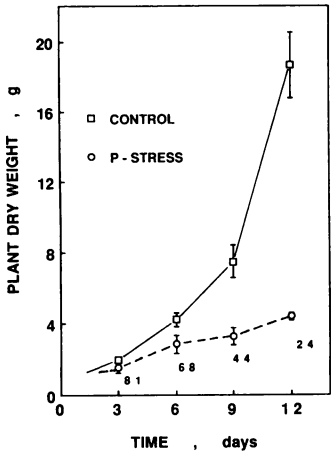

Figure 1. Whole-plant dry weight of control plants and plants deprived of an external P supply for 12 d. Inset values represent dry weight of −P plants expressed as a percentage of the control on each sample date. Standard error bars are included when larger than the symbol.

plants were removed from the solutions in which they were growing and placed into an identical solution with the exception that the 0.1 mM KH_2PO_4 was omitted. The other 16 plants remained in the control solution. The nutrient treatments were maintained for 12 d, as the plants without a P supply progressively became P stressed to a greater degree. At the beginning of light periods on days 3, 6, 9, and 12, four plants from each treatment were moved into solutions with the same composition but with $^{15}NO_3^-$ substituted for $^{14}NO_3^-$. After 12 h, at the end of the light period, the plants were harvested and shoots and roots frozen promptly at −20°C.

Tissue Analysis

The plant tissues were freeze dried, weighed, and ground. The tissues then were analyzed for various N fractions and P, and root tissues also were analyzed for carbohydrates.

The tissues were analyzed for NO_3^-, NH_4^+, SRN[3], individual amino acids, and IRN. Tissue was extracted with methanol:chloroform:water (13:4:3, v/v/v). Following separation of the chloroform from the methanol:water fraction, the chloroform was added back to the tissue residue, with this constituting the IRN fraction. Total nitrogen in the IRN fraction was determined by Kjeldahl digestion (16) and colorimetric analysis of NH_4^+ (3). An aliquot of NH_4^+ from the remaining digest was diffused into 0.5 N HCl and the atom percent ^{15}N determined mass spectrometically using a freeze-layer procedure (31).

With the methanol-water fraction, after the methanol was evaporated, an aliquot was removed and NO_3^- determined using a manual modification of the method of Lowe and Hamilton (14). The atom percent ^{15}N of the NO_3^- fraction was determined by mass spectrometry using a nitric oxide procedure (32). Other aliquots were removed from the methanol-water fraction and analyzed for individual amino acids and NH_4^+. Analysis of amino acids involved the use of Pico-Tag (Waters Assoc., Milford, MA) and HPLC (1). The NH_4^+ analysis involved colorimetric measurement of NH_4^+ (3) following a diffusion procedure to separate NH_4^+ from the tissue extract (refer to Rufty *et al.* [23]). The ^{15}N in individual amino acids and NH_4^+ was not determined. Nitrate in the remaining methanol/water fraction was volatilized by addition of H_2O_2 and H_2SO_4 (18), and then the SRN and atom percent ^{15}N were determined as in analysis of the IRN.

Shoot and root tissues were analyzed for total P. Samples (50–100 mg) were ashed overnight at 500°C, then the ash was dissolved in 4 M HCl and diluted with redistilled water. Aliquots were analyzed for P by the ammonium-molybdate method (17).

Additional root tissue was extracted with 80% ethanol to separate soluble carbohydrates and starch. The supernatant was analyzed enzymatically for total sucrose and hexoses (10). The particulate fraction, containing starch, was suspended in 1.0 ml of 0.2 N KOH and placed in boiling water for 30 min. After cooling, the pH was adjusted to 5.5 with 200 μL of 1.0 N acetic acid. To each sample, 1.0 mL of dialyzed amyloglucosidase solution (from Aspergillus niger [Sigma], 70 units/mL in 50 mM Na-acetate buffer [pH 4.5]) was added and the tubes were incubated at 55°C for 15 min. After digestion, the tubes were placed in boiling water for 1 min, centrifuged, and the glucose in the supernatant was analyzed using hexokinase and glucose 6-P dehydrogenase (10).

RESULTS

Plant exposure to nutrient solution without P resulted in a distinct growth inhibition relative to controls, which became more pronounced with time (Fig. 1). Dry weights of P-limited plants were 81% of controls on d 3, and only 24% on d 12 (Fig. 1, insets) when the plants appeared to be rapidly senescing. Root growth was affected less by the P limitation than shoot growth (Fig. 2). Root dry weight accumulation was similar to controls through d 6 and decreased only to 77% of the control on d 9 (Fig. 2A), while shoot dry weight was decreased to 80% of the control on d 3 and 39% on d 9 (Fig.

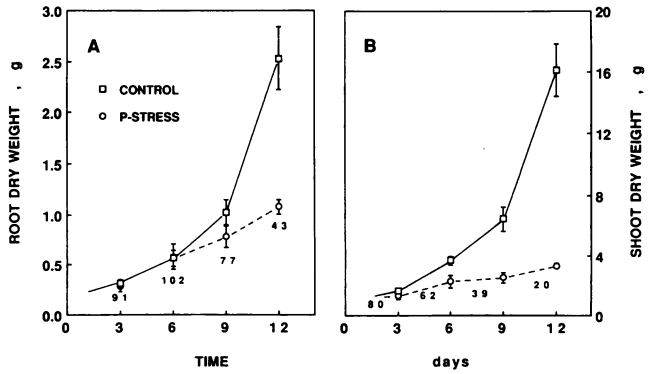

Figure 2. Dry weights of the root (A) and shoot (B) of control plants and plants deprived of an external P supply for 12 d. Inset values represent dry weights of −P plants expressed as a per cent of the control on each sample date. Standard error bars are included when larger than the symbol.

[3] Abbreviations: SRN, soluble reduced nitrogen; IRN, insoluble reduced nitrogen; NR, nitrate reductase.

Table I. *Concentrations of Soluble Sugars and Starch in the Root of Control Plants and Plants Deprived of an External Phosphorus Supply for 12 d*

Data are expressed as means ± SE.

Time	Soluble Sugars		Starch	
	−P	+P	−P	+P
d	*mg 100 mg^{-1} dry wt*			
3	3.42 ± 0.17	2.34 ± 0.12	0.36 ± 0.02	0.17 ± 0.03
6	3.38 ± 0.20	2.43 ± 0.16	0.34 ± 0.02	0.19 ± 0.01
9	1.96 ± 0.14	2.54 ± 0.24	0.31 ± 0.01	0.25 ± 0.01
12	2.17 ± 0.22	3.07 ± 0.16	0.27 ± 0.02	0.35 ± 0.03

2B). The differing root and shoot growth responses to P stress led to a steady decline in the shoot to root dry weight ratio from the 5.0 to 6.5 range for control plants to 4.5 on d 3 and 3.1 on d 12 (data not shown).

The concentration of soluble carbohydrates (sucrose and hexoses) and starch were elevated slightly in roots of P-stressed plants through d 6 (Table I). After that time, however, soluble carbohydrates declined to levels below those in roots of control plants, and starch levels were below those of controls on d 12.

The concentration of P in the shoot and root of P-stressed plants were noticeably lower than controls (Table II). In both plant parts, the P concentration quickly declined to and was maintained at about 20–30% of control levels.

P-stressed plants accumulated much lower amounts of N than control plants (Fig. 3). The decrease in N accumulation was evident by d 3 and became more pronounced with time, similar to decreases in whole-plant growth (*cf.* Fig. 1). Associated with lower N accumulation, the concentration of IRN (protein) was lower in the roots and shoots (Table III). The concentrations of NO_3^- and SRN (amino acids) were variable, but tended to be higher in the roots and shoot of P-stressed plants for much of the experiment. The largest concentration changes were those of NO_3^- in roots after d 3 and SRN in the shoot after d 6.

Individual components of the SRN fraction are shown in Table IV. The data represent mean values for NH_4^+ and amino acids over the last three sample dates when values were significantly altered relative to controls. The concentrations of NH_4^+ and the amino acids were similar in control and P-stressed plants on d 3. In the shoot of P-stressed plants after d 3, a majority of the increase in the SRN fraction (*cf.* Table III) was accounted for by increases in Asn, Gln, Pro, Gly, and NH_4^+. In roots of P-stressed plants, increases in Asn and Gln were evident. In contrast, in both the shoot and roots of P-stressed plants, Arg was noticeably lower than the controls.

On selected days following imposition of the P-stress treatment, plants were exposed to 1.0 mM $^{15}NO_3^-$ for 12 h periods to define alterations in NO_3^- assimilation in greater detail. Uptake of $^{15}NO_3^-$ g^{-1} root dry weight was substantially lower in P-stressed plants throughout (Fig. 4A). Uptake was 2.15 mmol g^{-1} compared with 3.07 mmol g^{-1} with the control on d 3, and declined to only 0.37 mmol g^{-1} compared with 2.26 mmol g^{-1} with the control on d 12. Whole-plant reduction of absorbed $^{15}NO_3^-$ also was inhibited in P-stressed plants, but the inhibition was delayed until d 6 (Fig. 4B). On d 9 and 12, about 30% of the absorbed $^{15}NO_3^-$ had been reduced at the end of the 12 h exposure compared to 60% in the control plants.

Alterations in endogenous $^{15}NO_3^-$ assimilation in P-stressed plants can be resolved further by examining the incorporation of ^{15}N into the different N fractions in each plant part (Figs.

Table II. *Concentrations of Total Phosphorus in the Shoot and Root of Control Plants and Plants Deprived of an External Phosphorus Supply for 12 d*

Data are expressed as means ± SE.

Plant Part	Time	Tissue Phosphorus	
		−P	+P
	d	*mg g^{-1} dry wt*	
Shoot	3	2.11 ± 0.16	6.32 ± 0.18
	6	1.33 ± 0.09	5.62 ± 0.42
	9	1.21 ± 0.09	5.96 ± 0.21
	12	0.93 ± 0.04	4.60 ± 0.20
Root	3	3.00 ± 0.27	9.31 ± 0.67
	6	2.19 ± 0.09	7.48 ± 0.18
	9	1.86 ± 0.04	7.47 ± 0.27
	12	1.51 ± 0.08	6.46 ± 0.23

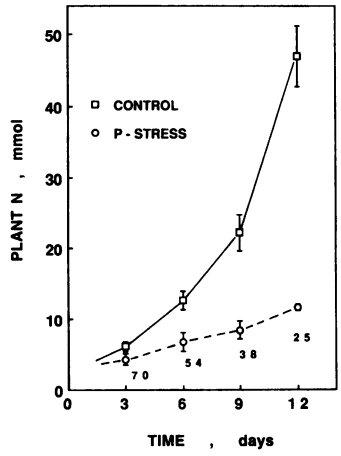

Figure 3. Total N (^{14}N + ^{15}N) accumulation by control plants and plants deprived of an external P supply for 12 d. Inset values represent total N of −P plants expressed as a per cent of the control on each sample date. Standard error bars are included when larger than the symbol.

Table III. *Nitrogen ($^{14}N + {}^{15}N$) Concentrations in Different Nitrogen Fractions in the Shoot and Root of Control Plants and Plants Deprived of an External Phosphorus Supply for 12 d*

Plant Part	Time	NO_3^-		SRN		IRN	
		−P	+P	−P	+P	−P	+P
	d			*mg 100 mg^{-1} dry wt*			
Shoot	3	0.69	1.00	0.42	0.39	2.76	3.02
	6	0.47	0.81	0.45	0.39	2.31	3.04
	9	0.75	0.85	0.64	0.29	2.14	3.03
	12	0.82	0.55	0.81	0.32	2.15	2.58
Root	3	0.81	1.44	0.38	0.23	2.68	2.72
	6	1.40	1.17	0.21	0.17	2.00	2.40
	9	1.55	1.07	0.28	0.20	1.91	2.96
	12	1.48	1.14	0.22	0.26	1.74	2.84

5 and 6). Treatment effects are seen most clearly when data are expressed as a percentage of the total ^{15}N available for assimilation, *i.e.* normalized for differences in $^{15}NO_3^-$ uptake. In roots of P-stressed plants, a much larger proportion of the absorbed ^{15}N label accumulated as $^{15}NO_3^-$ relative to controls (Fig. 5A). The increase was evident by d 3 and approached a maximum on d 6. Incorporation of ^{15}N into the IRN fraction was decreased, but the effect was not large until d 12 (Fig. 5C). In the shoot of P-stressed plants, there was no consistent change in the accumulation of $^{15}NO_3^-$; however, the proportion of absorbed label in the SRN and IRN fractions was decreased considerably after d 3 (Fig. 6).

Alterations in ^{15}N assimilation in the shoot also can be evaluated by expressing data as a percentage of the total ^{15}N in the shoot, *i.e.* normalized for differences in apparent translocation of ^{15}N to the shoot (Fig. 7). The incorporation of ^{15}N into shoot protein was noticeably decreased by P-stress after d 3 (Fig. 7C), whereas the proportion accumulating as $^{15}NO_3^-$ increased (Fig. 7A). The proportion of shoot ^{15}N in the SRN fraction was similar, in general, in P-stressed and control plants throughout the experiment.

Table IV. *Concentrations of Amino Acids and NH_4^+ ($^{14}N + {}^{15}N$) in the Shoot and Root of Control Plants and Plants Deprived of an External Phosphorus Supply for 12 d*

The data represent mean concentrations for the harvests on d 6, 9, and 12.

Plant Part	Nitrogen	Treatment	
		−P	+P
		μg 100 mg^{-1} dry wt	
Shoot	Asp	12.72	8.08
	Asn	50.36	6.64
	Gln	68.30	38.44
	NH_4^+	121.70	45.04
	Pro	33.82	13.79
	Trp	3.01	0.31
	Ser	15.73	8.14
	Gly	60.92	14.69
	Ile	2.39	0.61
	Leu	2.08	0.81
	Phe	2.39	1.39
	Tyr	1.51	0.53
	Arg	20.76	47.63
Root	Asn	7.06	1.36
	Gln	10.24	3.83
	Arg	19.65	38.60

DISCUSSION

Our results indicate that there are multiple alterations in the NO_3^- assimilation pathway in plants experiencing P stress. Exposure of plants to labeled $^{15}NO_3^-$ revealed that NO_3^- uptake into the root from the external solution, translocation of absorbed NO_3^- out of the root to the shoot, and NO_3^- reduction in the shoot all were restricted. The changes in NO_3^- transport in the root are consistent with observations from previous studies (7, 13, 25).

Transport Effects

Nitrate uptake was particularly sensitive to the P limitation. Uptake of $^{15}NO_3^-$ g^{-1} root dry weight decreased to 70% of the control by d 3, and to 30% on d 6 (Fig. 4A). The uptake response could have resulted from a number of factors associated with the P-stress condition. One obvious possibility is

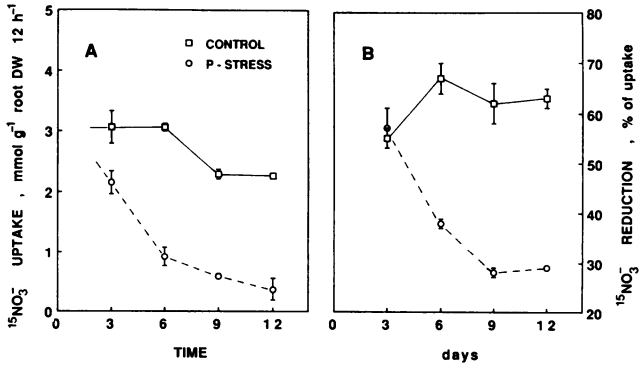

Figure 4. Uptake of $^{15}NO_3^-$ g^{-1} root dry weight (A) and whole-plant $^{15}NO_3^-$ reduction (B) of control and −P plants exposed to $^{15}NO_3^-$ for 12 h on selected days during the 12-d experiment. The $^{15}NO_3^-$ reduction values represent the sum of $SR^{15}N$ and $IR^{15}N$ in the whole plant expressed as a percentage of the plant ^{15}N. Standard error bars are included when larger than the symbol.

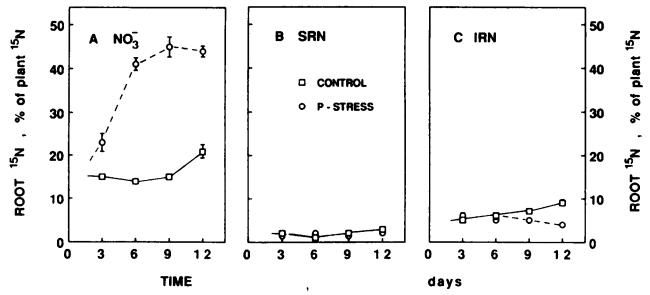

Figure 5. Alterations in the distribution of ^{15}N among nitrogen fractions in the root of control and −P plants exposed to $^{15}NO_3^-$ for 12 h on selected days of the experiment. Data are expressed as a percentage of the total ^{15}N in the plant. Standard error bars are included when larger than the symbol.

decreased availability of energy (ATP), required for active uptake of NO_3^- across the plasma membrane of root cells (6). Root growth, however, was similar to the control until after d 6 (Fig. 2A), and root carbohydrate levels were maintained slightly higher than controls during the same time interval (Table I). Moreover, there was no indication that assimilation of absorbed $^{15}NO_3^-$ into organic molecules was being inhibited until after d 6 (Fig. 5, B and C). The evidence is thus inconsistent with a general energy limitation in the root causing the decline in $^{15}NO_3^-$ uptake in the first week of treatment. Decreased energy availability may well have contributed to the uptake restriction after d 6.

On the other hand, the possibility that a specific regulatory effect was exerted on the formation or activity of the NO_3^- transport system in the first week of P stress would appear plausible. This could involve a direct response to low P status in the root (Table II), or it could be an indirect consequence of altered N relations. Elevated concentrations of NO_3^- (Table III) and the amino acids Asn and Gln (Table IV) were measurable by d 6, which could be indicative of feedback inhibition of NO_3^- uptake (2, 4, 29).

An accumulation of $^{15}NO_3^-$ in the root was evident upon first exposure to $^{15}NO_3^-$ on d 3 (Fig. 5A). Thereafter, a larger proportion of absorbed $^{15}NO_3^-$ was retained in roots of P-stressed plants relative to controls. Restricted translocation could involve inhibition of NO_3^- transport out of the root symplasm into the xylem, across the plasma membrane of xylem parenchyma cells (*cf.* 8, 19), and/or stimulation of NO_3^- transport into cell vacuoles in the root cortex and stele. An association with altered water relations is conceivable. The hydraulic conductivity of the root system decreases within several days after plants are deprived of an external P supply (22), which could lead to altered tonoplast and plasmalemma transport activities, including those involved in uptake (5).

The NO_3^- transport responses in roots of P-stressed plants resemble changes in P transport which occur when plants are deprived of N. Lower P uptake rates and rates of P translocation into the xylem were evident in laboratory and field experiments with corn (11, 12, 26, 27). It would appear that the reciprocal interactions represent a sensitive control mechanism localized in the root which ensures that the supply of P and N for plant metabolism remains in balance.

Endogenous Assimilation

The retention of $^{15}NO_3^-$ substrate in the root was the main endogenous event limiting whole-plant $^{15}NO_3^-$ reduction efficiency (Fig. 4B) and ^{15}N incorporation into leaf IRN (protein) once $^{15}NO_3^-$ was absorbed by P-stressed plants (*cf.* Figs. 5 and 6). The accumulated $^{15}NO_3^-$ escaped reduction even during the first 6 d of treatment when energy relations in the root appeared unimpaired. Compartmentation of $^{15}NO_3^-$ spacially separate from NR is implied (9, 15, 24). In addition to the restricted delivery of NO_3^- substrate from the root, shoot protein synthesis apparently was limited in the P-stress condition by decreased reduction of available NO_3^- in shoot tissues, as the proportion of shoot ^{15}N accumulating as unassimilated $^{15}NO_3^-$ increased noticeably above controls after d 3 (Fig. 7A). The cause is unknown; decreased NR activity, compartmentation of $^{15}NO_3^-$ or energy limitations on reduction could have been involved.

It is somewhat surprising that there was no indication of a buildup of ^{15}N in the SRN (amino acid) fraction in leaves of P-stressed plants. As mentioned previously, numerous studies have reported elevated amino acid levels in leaves of P-stressed plants, and higher concentrations of total SRN (Table III) and

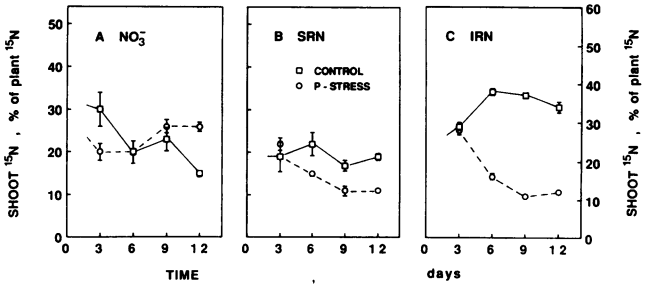

Figure 6. Alterations in the distribution of ^{15}N among nitrogen fractions in the shoot of control and −P plants exposed to $^{15}NO_3^-$ for 12 h on selected days of the experiment. Data are expressed as a percentage of the total ^{15}N in the plant. Standard error bars are included when larger than the symbol.

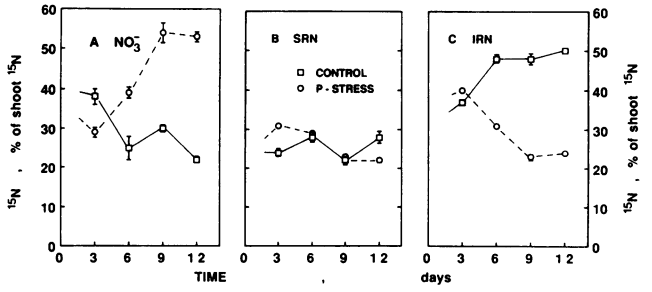

Figure 7. Distribution of shoot ^{15}N among nitrogen fractions in control and −P plants exposed to $^{15}NO_3^-$ for 12 h on selected days of the experiment. Standard error bars are included when larger than the symbol.

individual amino acids (Table IV) were evident here in shoot tissues after d 3. The absence of an accompanying buildup of ^{15}N in the SRN fraction indicates (a) that the higher accumulations of amino acids originated in large part from degradation of protein formed prior to the exposure periods (*cf.* 30), and (b) that the accumulated amino acids were sequestered in a compartment (older leaves?) separate from that supplying concurrent protein synthesis.

In experimentation with citrus species, Rabe and Lovatt (20, 21) found concentrations of Arg to be elevated in leaves of P-stressed plants. It was proposed that enhanced Arg synthesis was a protective mechanism minimizing toxicity effects associated with the excessive accumulation of NH_4^+. In our experiment, concentrations of NH_4^+ were higher in the shoot of P-stressed plants but concentrations of Arg were lower (Table IV), suggesting a different type of response system. It is unknown to what extent the sensitivity of tobacco to P stress or NH_4^+ toxicity differs from that of citrus or other species.

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